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Evasion of alternative complement pathway by *Trypanosoma cruzi* results from inefficient binding of factor B

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ABSTRACT **During its differentiation in the insect vector to a stage infective for the mammalian host, *Trypanosoma cruzi* becomes resistant to lysis by the alternative pathway of complement. To elucidate the mechanism of complement evasion, we studied control of complement activation on the surface of the noninfective epimastigote and the infective culture-derived metacyclic trypomastigote stages (CMT) of *T. cruzi*. It was found that the predominant form of complement component C3 on epimastigotes is C3b, whereas the majority of C3 on CMT is in the form of the hemolytically inactive fragment iC3b, which cannot participate in C5 convertase formation or lead to deposition of the lytic C5b-9 complex. Our results also showed that C3 binds by a covalent ester linkage to surface molecules of different molecular weight in the epimastigote stage and CMT. Binding studies with purified complement components indicated that CMT do not support efficient formation of an alternative pathway C3 convertase. C3b on the parasite surface fails to bind the amplification component, factor B, rather than showing enhanced binding of the control component, factor H. These results identify the biochemical basis for evasion of complement-mediated killing in *T. cruzi* and reveal a mechanism for developmental regulation of complement activation.**

Protozoan parasites have developed a variety of mechanisms to evade the immunologic defense mechanisms of the mammalian host. Thus, vertebrate-stage parasites and infective vector-stage parasites typically resist direct serum killing and can also evade ingestion or intracellular killing by the phagocytic cells of the host. In contrast, most noninfective vector-stage parasites are susceptible to direct lysis by serum and to phagocytosis and destruction by monocytes or macrophages. For example, the epimastigote stage (Epi) of *Trypanosoma cruzi*, which multiplies in the gut of the vector, is efficiently lysed in human serum by means of alternative complement pathway (ACP) activation (1). In contrast, the infecting metacyclic trypomastigote stage, the amastigote stage (which multiples intracellularly in the vertebrate host), and the disseminating bloodstream trypomastigote form are not lysed when incubated in human serum (1–3). The membrane changes that control the transformation of these parasites from activators of the ACP to nonactivators are incompletely understood, as are the molecular interactions of the ACP with the parasite surface of various life-cycle stages.

Activation of the ACP is currently thought to be mediated and regulated by the following mechanisms: continuous low-grade fluid-phase generation of complement component C3b or water-hydrolyzed C3 occurs normally in serum and results in the random deposition of C3b on all particles (4). The subsequent interaction of this randomly deposited C3b with proteins of the ACP is determined by the nature of the particle surface (5–7). On alternative pathway activators, C3b binds factor B in preference to factor H. C3b that bears B cannot interact with the inactivator enzyme, factor I. Rather, B bound to C3b is cleaved by factor D to produce the active enzyme Bb. The complex of C3b–Bb cleaves additional C3, producing C3b, which leads to amplification of C3b deposition on the activator surface. In contrast, on nonactivators, H (rather than B) is more likely to be bound to C3b. The complex of C3b bearing H does not interact with D. Instead, H acts as a co-factor for I in the cleavage of C3b, yielding the inactive fragment iC3b, which cannot participate in further amplification of C3b deposition.

We have studied (8) the interaction of the ACP with Epi and with culture-derived infecting metacyclic trypomastigotes (CMT) (9). Our results showed that 5-fold more C3 is deposited on Epi than on CMT when these parasites are incubated in normal human serum. Furthermore, GP72, a major developmentally regulated surface glycoprotein, is the preferential site for C3b binding (C3 acceptor) on Epi, but despite its presence it is a poor acceptor for C3 binding on CMT. Since the environment in which C3b is deposited influences its interaction with other complement proteins, the difference in extent of C3 deposition and in the C3 acceptor on Epi and CMT suggests that C3b molecules may be metabolized differently on these life-cycle stages.

In this paper, we have examined the structure of C3 on both Epi and CMT, as well as the mechanism by which alternative pathway convertase formation is controlled in these two life-cycle stages. Our results show that CMT do not support efficient formation of an alternative pathway C3 convertase because CMT fail to bind factor B. Unexpectedly, both Epi and CMT bind the control component factor H with high affinity.

MATERIALS AND METHODS

Buffers and Reagents. The following buffers were used in these experiments: Hanks' balanced salts solution containing 0.15 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 2 mg of human serum albumin per ml ($HBSS^{++}$-A); lysis buffer, containing 0.05 M Tris (pH 8.2), 100 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.05% sodium azide; lysis buffer containing 1 M NH_2OH (pH 10.5); and isotonic Veronal-buffered saline containing 4 mM $MgCl_2$ and 2 mg of human serum albumin per ml (VBS^{+}-A).

***T. cruzi* Strains and Clones.** The Tulahuen strain, the WA250 clone, and the Miranda 88 clone of *T. cruzi* (M88) were obtained from J. Dvorak (National Institutes of Health, Bethesda, MD). The Epi of these isolates were maintained by serial passage in liver-infusion tryptose broth, as described (8). CMT of the M88 clone were produced with a modification of Sullivan's method (9) and separated from the Epi with an

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Abbreviations: ACP, alternative complement pathway; CMT, infecting metacyclic trypomastigote stage; Epi, epimastigote stage; ^{125}I-C3, ^{125}I-B, and ^{125}I-H, ^{125}I-labeled complement component C3, factor B, and factor H; PNHS, pooled normal human serum.

adaptation of the procedure of Lanham and Godfrey (10). The final suspensions contained >95% CMT.

Serum. Pooled normal human serum (PNHS) was collected from eight normal volunteers and frozen in aliquots at −70°C. Serum was also obtained from a patient with a congenital deficiency of C8, and it was frozen in aliquots at −70°C.

Complement Component Purification. Human complement components C3, B, and C9 were purified with minor modifications of the procedure described by Hammer *et al.* (11). Factor H (12) and factor D (13) were isolated as reported earlier. C3 nephritic factor was kindly provided by Robert McClean (Johns Hopkins University, Baltimore, MD).

C3, C9, B, and H were labeled with radioiodinated sodium (^{125}I-Na) (New England Nuclear) using Iodobeads (Pierce) to specific activities of 3.2×10^5 cpm/μg, 7.4×10^5 cpm/μg, 8.8×10^5 cpm/μg, and 7.67×10^5 cpm/μg, respectively. C3 was also labeled with [^{3}H]$NaBH_4$ (New England Nuclear) by the process of reductive methylation to a specific activity of 1.14×10^5 cpm/μg.

Form of C3 on Epi and CMT During Incubation in Serum. Epi of the Tulahuen strain and the WA-250 and M88 clones and CMT of the M88 clone were incubated for 60 min at 37°C at a concentration of 5×10^7 parasites per ml in 20% C8-deficient serum in HBSS^{++}-A containing ^{125}I-C3. At various times, aliquots were removed and washed three times in HBSS^{++}-A. The washed pellets were solubilized in 1% $NaDodSO_4$ at 100°C for 5 min. The detergent-soluble supernatant (which contained >90% of the bound ^{125}I-C3) was collected after a 5-min centrifugation at 12,500 × *g* and was then diluted 1:20 with 1 M NH_2OH (pH 10.5) in lysis buffer to cleave the covalent ester linkage between the C3 and acceptor molecules (8, 14). The resultant mixture was processed as described (15) and C3 fragments were analyzed by 10% $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography.

C9 Binding to Epi and CMT. Binding of C9 to Epi and CMT was measured during incubation in 20% PNHS in HBSS^{++}-A containing ^{125}I-C9, using the reaction conditions described (8).

Deposition of C3 on Epi and CMT with Purified B, D, and C3. Epi and CMT, at 5×10^7 per ml in VBS$^+$-A, were incubated for 60 min at 37°C with 400 μg of C3, 14 μg of [^{3}H]C3, 51 μg of B, 0.3 μg of D, and 30 μg of C3 nephritic factor. The parasites were washed once and then subjected to two sequential cycles of amplification. Each cycle consisted of a 20-min incubation at 30°C with 51 μg of B and 0.3 μg of D, followed by centrifugation at 4°C and addition of 400 μg of C3 and 14 μg of [^{3}H]C3 to the pellet for an additional incubation at 37°C for 30 min. The total number of molecules of bound C3b was determined at each step as reported (8). For experiments in which the molecular form of deposited C3 was to be examined, 100 μg of ^{125}I-C3 was used instead of [^{3}H]C3 during the second amplification step.

Binding of ^{125}I-B and ^{125}I-H to Epi- and CMT-Bearing C3b. Complement component C3b was deposited on Epi and CMT by using sequential cycles of amplification with B and D and C3, as described above. The Epi and the CMT, both bearing C3b, were incubated for 20 min at 30°C at 5×10^6 per ml with dilutions of either ^{125}I-B or ^{125}I-H in VBS$^+$-A. The number of bound molecules was then determined as described (8). Nonspecific binding of ^{125}I-B and ^{125}I-H was measured concomitantly on the C3b-bearing Epi and CMT by addition of a 50- to 100-fold excess of unlabeled B or H or by binding of ^{125}I-B and ^{125}I-H to the Epi and CMT not bearing C3b. The binding of ^{125}I-B and ^{125}I-H was plotted according to the method of Scatchard (16). The binding data were subjected to a nonlinear least-squares curve fitting (17) using a generalized model for complex ligand–receptor systems as described (18).

RESULTS

Molecular Form of C3 on Epi and CMT. To study the metabolism of C3 on the surface of Epi and CMT, we examined the C3 fragments on these life-cycle forms after serum incubation. Intact C3 is a two-chain molecule, containing an α chain of 120 kDa disulfide-linked to a β chain of 75 kDa (19). Cleavage of C3 to C3b results in cleavage of the α chain to a 110-kDa α' chain, which binds covalently to particle surfaces. Further cleavage of the α' chain to 68- and 46-kDa fragments produces $iC3b_1$.

On three different strains of *T. cruzi* Epi, all of which activate the ACP, the predominant form of C3 observed after serum incubation was C3b containing the 110-kDa α'-chain fragment and the 75-kDa β chain (Fig. 1). Also apparent in lesser amounts was a 68-kDa fragment from the α' chain, which is consistent with further cleavage of the C3b molecule to iC3b. The α' chain representative of C3b predominated by 3 to 1 over the 68-kDa fragment indicative of iC3b, showing that approximately three-fourths of bound C3 was present as C3b. The results were strikingly different with the CMT of *T. cruzi*, which activated the ACP inefficiently. As was apparent after both a 20- and a 60-min incubation in serum, the major form of C3 was iC3b. On CMT, the 68-kDa α'-chain fragment of iC3b predominated by 6- to 9-fold over the 110-kDa α'-chain fragment of C3b.

Deposition of C9 on Epi and CMT. We next examined whether the difference in C3 fragmentation and extent of C3 deposition on Epi and CMT was reflected in a difference in extent of terminal complement deposition. Deposition of C9 on Epi and CMT was determined during incubation in 10% and 40% PNHS. Between 4- and 6-fold more C9 bound to Epi than to CMT (Table 1). Therefore, C9 deposition paralleled C3 deposition.

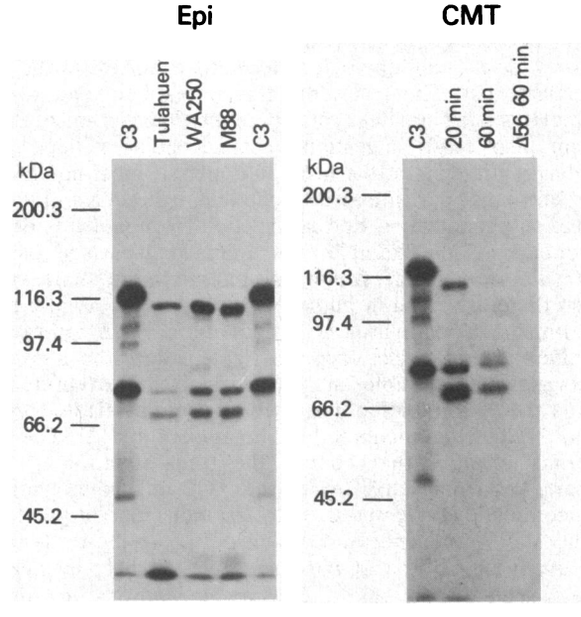

FIG. 1. Form of C3 on Epi and CMT during incubation in serum. Epi of the Tulahuen strain and the WA-250 and M88 clones, and CMT of the M88 clone were incubated at 37°C in C8-deficient serum containing ^{125}I-C3. Epi samples were examined after 60 min of incubation; CMT samples were processed after both 20-min and 60-min incubation periods. Shown above is a 10% $NaDodSO_4$/PAGE autoradiogram revealing the C3 fragmentation pattern on Epi and CMT after solubilization in 1% $NaDodSO_4$ and treatment with hydroxylamine. The absence of material at the top of the autoradiogram indicates that all of the bound C3 has been released from acceptor molecules by hydroxylamine.

Table 1. Binding of C9 to Epi and CMT during incubation in PNHS

	C9 molecules per parasite, $\times 10^{-5}$		
	10% PNHS	20% PNHS	40% PNHS
Epi	3.20 ± 0.58	7.36 ± 0.21	7.93 ± 2.61
CMT	0.78 ± 0.19	1.35 ± 0.16	1.88 ± 0.33

Parasites at 5×10^7 per ml were incubated for 60 min in 10%, 20%, or 40% PNHS containing ^{125}I-C9. Molecules of C9 bound per parasite were determined as described (8). Results shown are mean ± SD for two experiments, each done in duplicate.

Deposition of C3b on Epi and CMT with Purified B, D, and C3. We next pursued the biochemical basis for the difference in C3 fragmentation on Epi and CMT. This was approached by examining how the C3b on the parasite surface interacts with B and H. To prevent C3b from converting to iC3b when the molecule is deposited on Epi and CMT, we used purified amplification proteins of the alternative pathway in the absence of the inactivating proteins H and I. This procedure permits the deposition of C3b on both activators and nonactivators of the alternative pathway since regulation of amplification by conversion of C3b to iC3b cannot occur. The initial deposition of C3 on the parasite surface was accomplished with a fluid-phase alternative pathway convertase; further C3 binding was attained with two sequential cycles of amplification. Initial deposition of C3b on the surface of Epi and CMT was equivalent, consistent with the random deposition of fluid-phase-generated C3b on the parasite surface (Table 2). In subsequent cycles, more C3b was deposited on Epi than on CMT, with the result that after the third cycle, 2½- to 4-fold more C3b was deposited on Epi than on CMT. Furthermore, increases in C3b deposition with each cycle were less for CMT than for Epi. These results suggest that C3 convertase formation is less efficient on CMT than on Epi.

Nature of the Bond and Stability of C3b on Epi and CMT. Before continuing with the investigation of the interaction of C3b on the parasite surface with B and H, we examined some characteristics of the C3b deposited with the purified alternative pathway proteins. First, we showed that the C3b deposited both on Epi and on CMT was attached by a covalent ester bond to parasite molecules, as indicated by the retarded migration of the α' chain of C3b on NaDodSO$_4$/PAGE and by hydroxylamine release of the α' chain from its acceptor molecule (Fig. 2). The migration of the α'-acceptor complex was different on Epi and CMT. The predominant band for Epi migrated at 180–185 kDa, consistent with covalent attachment of the α' chain to GP72. In contrast, the major band on CMT migrated at 135–140 kDa, suggesting that the α' chain was bound to a molecule of 25–30 kDa. Next, the stability of C3b on the surface of Epi and CMT was tested. For parasites held at 37°C for 3 hr or at 4°C for 2 days after deposition of C3b, there was no evidence of cleavage of the α' chain, arguing against the conversion of C3b to iC3b or other fragments by a parasite-derived protease (data shown only for CMT at 3 hr). The bands migrating at ≈280 kDa (the origin of the gel) in the Epi lanes and the non-hydroxylamine-treated CMT samples represent either α' chain that is ester linked to a large parasite acceptor molecule or aggregated C3 that dissociates with hydroxylamine treatment.

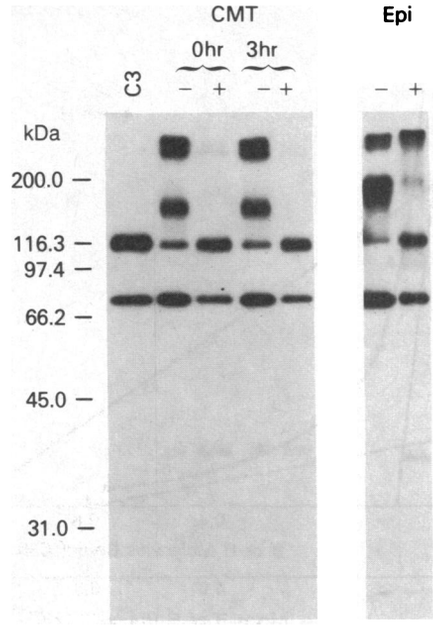

FIG. 2. Characteristics of the C3b acceptor bond on Epi and CMT. ^{125}I-C3b was deposited on Epi and CMT by using purified amplification proteins of the ACP. Bound ^{125}I-C3 fragments were then analyzed by 10% NaDodSO$_4$/PAGE before (−) and after (+) treatment with 1 M hydroxylamine to release covalent ester linkages. For CMT, the C3 fragmentation pattern is also shown for parasites incubated at 37°C for an additional 3 hr after completion of ^{125}I-C3b deposition.

Binding of ^{125}I-H and ^{125}I-B to C3b-Bearing Epi and CMT. We next examined the interaction of purified radiolabeled B and H with parasite-bound C3b. Saturation binding studies of ^{125}I-B and ^{125}I-H were performed along with ligand binding analyses to determine the affinity of B and H for C3b and the maximum number of B and H binding sites per C3b residue (Fig. 3). For Epi (Fig. 3*A*), the linear curve for B binding indicated that there was a single population of B binding sites, whereas H binding was most consistent with two classes of H binding sites (dashed lines). The single class of B binding sites was of lower affinity than the high-affinity H binding site (represented by the steep line), but the average B affinity for C3b was higher than the low-affinity H binding site (represented by the shallow line). It is apparent from inspection of the x intercepts that the number of B binding sites with an affinity exceeding that of H binding sites was substantial, although both B and H recognized the majority of bound C3b. Thus, on Epi, alternative pathway activation occurred be-

Table 2. Deposition of C3b on Epi and CMT with B, D, and C3

	Epi		CMT
Cycle	Tulahuen	M88	M88
Initial	0.19 ± 0.03*	0.22 ± 0.04	0.19 ± 0.01
Second	1.90 ± 0.36 (9.4)†	1.35 ± 0.11 (6.1)	0.74 ± 0.17 (3.9)
Third	15.26 ± 0.91 (7.7)	11.06 ± 0.82 (8.2)	3.88 ± 0.53 (5.2)

Initial deposition of complement component C3b on Epi and CMT was accomplished by using a fluid-phase convertase containing C3 nephritic factor. Subsequent cycles of amplification of particle-bound C3b were accomplished by incubation with B and D, followed by addition of C3.
*Molecules of C3 per parasite ($\times 10^{-5}$) ± SEM for three experiments.
†Fold increase over previous step.

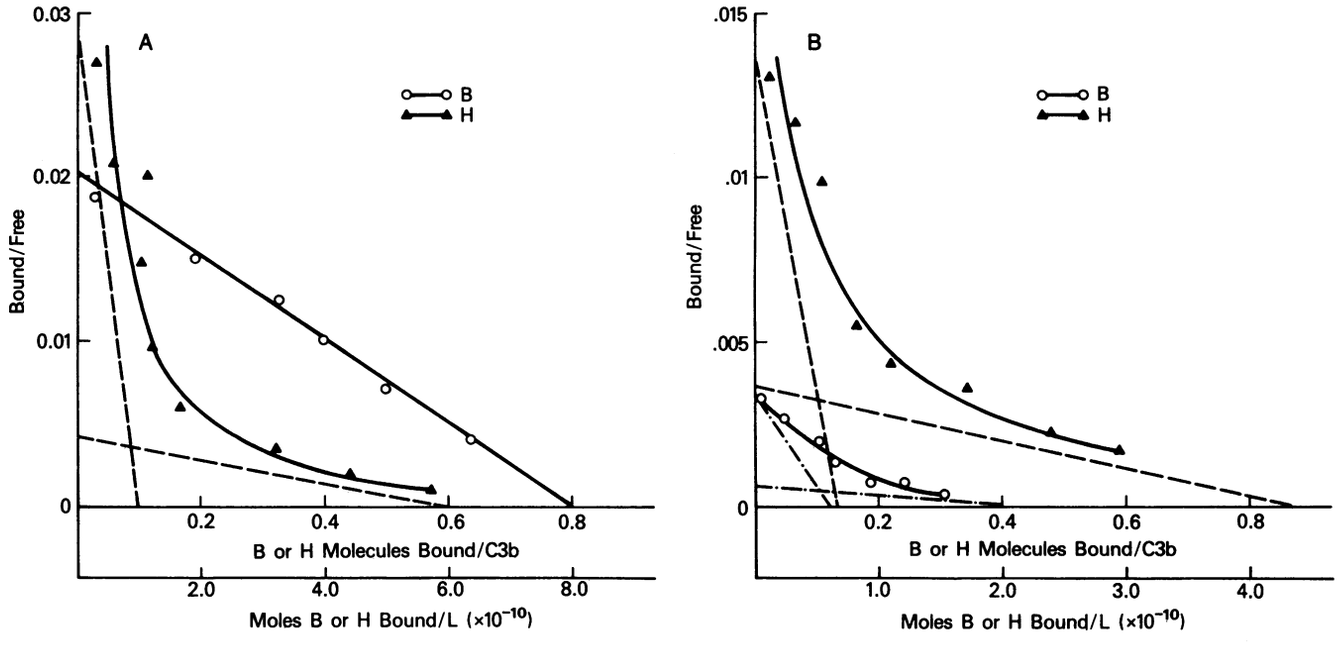

FIG. 3. Complement component C3b was deposited on Epi and CMT by using the purified ACP proteins B, D, and C3. Then, saturation binding studies were done with ^{125}I-B and ^{125}I-H, and the binding data were subjected to a nonlinear least-squares curve fitting (17) using a generalized model for complex ligand–receptor systems as described (18). For the C3b on Epi(A), the data were most consistent with a single population (K_a = 1.3 × 10^7 liter/mol) of B binding sites (○) and two classes (K_a = 1.56 × 10^8 liter/mol; 3.5 × 10^6 liter/mol) of H binding sites (– – –). For the C3b on CMT (*B*), there were two classes of binding sites for B (K_a = 3.0 × 10^7 liter/mol; 1.7 × 10^6 liter/mol; –·–) and H (K_a = 1.15 × 10^8 liter/mol; 4.4 × 10^6 liter/mol; – – –).

cause, on balance, the binding of B to C3b was favored over the binding of H to C3b.

The results were substantially different with CMT (Fig. 3*B*). Scatchard plots of both B and H binding (solid lines) were curvilinear, and the results of ligand binding analysis were consistent with that of two classes of binding sites for both B and H (dashed lines). The number of high-affinity binding sites for B was not different from that for H, and there were fewer low-affinity binding sites for B than for H. Furthermore, only 55% of the C3b on CMT was bound by B, in comparison to full recognition of the C3b on CMT by H. Thus, when Epi and CMT were compared, the major difference between the binding profiles of H and B was a decrease in the extent of B binding on CMT. These results are similar to those reported for rough and type 7 *Streptococcus pneumoniae* (20) but are unlike those seen in most other ACP activator/nonactivator (5–7, 21, 22) systems, in which differential H binding (often dictated by the amount of sialic acid present) controls the extent of activation.

DISCUSSION

Results from this study show that infective culture derived metacyclic trypomastigotes of *T. cruzi* do not support efficient formation of an alternative pathway C3 convertase, because they fail to bind the alternative pathway amplification component, factor B. In contrast, they bind the control component, factor H, with high affinity. As a consequence, the majority of C3 on CMT is cleaved, producing the hemolytically inactive fragment iC3b, which cannot participate in formation of a C5 convertase or lead to C5b-9 deposition. This conclusion is evidenced by the deposition of fewer C9 molecules (by a factor of 4–6) on CMT than on Epi. We have also found (M. T. Rimoldi and K.J., unpublished observations) that less C3 and C9 (by a factor of 6–8) is deposited on tissue culture-derived trypomastigotes (TCT) than on Epi, and that the major form of C3 on TCT is iC3b. This result indicates that the findings reported here for CMT may apply as well to vertebrate stage parasites of *T. cruzi*.

There are at least three general mechanisms that may explain the differences between Epi and CMT in terms of B binding to C3b. First the protein or carbohydrate portion of GP72 may be altered on CMT to render the molecule a poor C3 acceptor in addition to being one that disfavors B binding to C3b. A second possibility is the production of another molecule in CMT, which blocks C3 binding to GP72. This molecule could either serve as the C3 acceptor itself or redirect C3 deposition to another site at which poor B binding to C3b occurs. Finally, CMT may produce a molecule that either interferes with the binding of B to C3b or accelerates decay of the C3b–Bb complex (23–27). Preliminary evidence exists supporting each of these possibilities. Structural studies indicate that GP72 is altered in CMT compared to Epi (8), and that new surface-exposed glycoproteins appear in CMT during metacyclogenesis (28, 36). In addition, data in Fig. 2 suggest that the major C3 acceptor in CMT is a molecule of 25–30 kDa, whereas the C3 acceptor in Epi is GP72 (8). Finally, recent experiments in our laboratory suggest that CMT contain a molecule capable of accelerating decay of the alternative pathway C3 convertase. Regardless, the capacity of protease and endoglycosidase treatment to render CMT susceptible to complement lysis and to increase total C3 deposition and the fraction of C3b molecules on the parasite surface (36) suggests that control is exerted by a cell-surface glycoprotein.

A variety of mechanisms are described for evasion of complement-mediated killing by microorganisms and nucleated cells. Complement resistance may result from inefficient complement activation, from a block in the cascade after initiation of the sequence but before C5b-9 formation, or from internalization or shedding of the fully formed C5b-9 complex (29–31). In some circumstances, a C5b-9 complex forms and remains stably attached on the cell membrane without causing lethal damage, either because the complex is not deposited at lethal sites or because the metabolic pathways of the

cell compensate for ion flux through the C5b-9 channel (32). In *T. cruzi*, our results show that inefficient activation is a primary means of complement resistance for CMT. However, we cannot exclude an additional mechanism of resistance, such as deposition of C5b-9 on locations that are ineffective for parasite lysis.

Other protozoa (*Leishmania* and African trypanosomes) become resistant to ACP lysis during differentiation to infective stages (33, 34). The infective or vertebrate stage can often be rendered susceptible to ACP lysis by protease treatment (2, 35), implying that an additional molecule that abrogates effective ACP activation has been removed. This hypothesis has yet to be formally proven and the question of whether B binding regulation is a general mechanism for complement evasion remains to be investigated.

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